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Inhibition of Acetylcholinesterase Activity in Human Brain Tissue and Erythrocytes by Galanthamine, Physostigmine and Tacrine

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Summary: Galanthamine, physostigmine and 9-amino-1,2,3,4-tetrahydroacridine (tacrine) were evaluated as inhibitors of human acetylcholinesterase activity from samples of postmortem human brain, fresh brain cortex biopsies and human erythrocytes. Acetylcholinesterase activity was most effectively inhibited in all tissues by physostigmine, followed by tacrine and galanthamine. The respective inhibitor concentrations exerting a half maximal effect (IC_{50}) on acetylcholinesterase in postmortem human brain frontal cortex were 14 nmol/l, 1.0 µmol/l and 3.2 µmol/l versus 15 nmol/l, 1.1 µmol/l and 2.8 µmol/l in the hippocampus region. In addition, the inhibition of acetylcholinesterase by galanthamine was similar in postmortem brain and brain cortical biopsies from patients submitted to brain-tumour removal, indicating that postmortem changes up to 28 h after death probably did not influence the measurement of acetylcholinesterase inhibition. While physostigmine and tacrine acted equally on acetylcholinesterase from different sources, galanthamine was 10-fold less potent in inhibiting the enzyme activity from human brain than from human erythrocytes. Comparison with issues from mice revealed that galanthamine was selectively more potent in suppressing acetylcholinesterase in human erythrocytes. The results are discussed in the light of pharmacokinetic data, and conclusions are drawn for further clinical studies.

Introduction

Reversible cholinesterase inhibitors are currently used for symptomatic treatment of cognitive deficits and memory impairment in *Alzheimer*'s disease. One postulated mechanism is restoration of the cholinergic deficit at synaptic sites in the brain by inhibition of acetylcholine metabolism (1), but several other mechanisms have also been discussed (2–5). It was the purpose of this study to measure the inhibition of acetylcholinesterase[1]) from various sources by galanthamine, physostigmine and tacrine. A series of concentration response trials was performed using human brain tissue; and, prior to further specified analysis currently in progress, two separate regions of interest in *Alzheimer*'s disease were determined: the frontal cortex and the hippocampus. Since measurement of red-cell acetylcholinesterase inhibition ex vivo may predict enzyme inhibition in the brain (6, 7), it seemed useful to perform additional in vitro experiments with erythrocytes. Postmortem acetylcholinesterase activity has been shown to be stable (8) for at least 31 h. It has also been reported, however, that the 4S and 10S molecular forms in the brain are extremely labile and that freezing of either subcellular or intact tissue causes dramatic shifts in the level of the molecular forms (9), although the molecular shift was not associated with any change of total acetylcholinesterase activity. To identify possible alterations in postmortem tissue, the inhibition of acetylcholinesterase by galanthamine in fresh human brain cortex samples

[1]) Enzymes: Acetylcholinesterase, EC 3.1.1.7

obtained at biopsies in 4 patients during brain-tumour ablation was also studied. In addition, the inhibitory effects of galanthamine, physostigmine and tacrine on acetylcholinesterase in vitro were also measured in erythrocytes and fresh brain tissue samples of animals.

Materials and Methods

Postmortem human brain tissue from the frontal cortex and hippocampal region of 11 individuals without evidence of psychiatric illnesses (7 female, 4 male, age 56–87) was dissected 5–28 h after death, frozen and stored at −70 °C until examination. Neuropathological examination of matching samples ensured that the patients showed no evidence of specific neurological illness. Fresh surgical samples of human cortex were obtained from 4 patients where such tissue was removed to gain access to the tumour (10). The non-tumorous (peritumorous) tissue was immediately frozen and stored as described above. Aliquots of laboratory routine whole blood samples were taken from the same patients prior to the surgical procedure, centrifuged and the red cells separated. Adult male NMRI-mice (bred by the Bundesgesundheitsamt Berlin and kept under standardized conditions) were killed by decapitation; trunk blood was collected in heparinized plastic tubes and mixed immediately. Whole blood was centrifuged, and the red cells were separated as above. Erythrocytes were haemolysed by freezing and thawing 3 times using liquid nitrogen. Brain samples were weighed and homogenized using an Ultra Turrax (Janke und Kunkel, Staufen, Germany) for 20 s at 20 000 min^{-1} in 3 volumes of ice-cold *Sörensen*'s phosphate buffer. The catalytic activity of acetylcholinesterase in erythrocytes and brain was measured as previously described in detail (11, 12), using [^{14}C]acetylcholine iodide (NEN, Dreieich, Germany) radiolabelled in the acetyl moiety at a final substrate concentration of 3.6 mmol/l, a pH of 7.4 and a temperature of 25 °C. Concentration response trials were performed as previously described (11, 12). The inhibitors used were galanthamine (Nivalin™, Waldheim, Vienna, Austria), tetraisopropylpyrophosphoramide (iso-OMPA, Sigma, Deisenhofen, Germany), physostigmine (Serva, Heidelberg, Germany) and tacrine (Serva, Heidelberg, Germany). After incubation of the sample with the inhibitor for 60 min at 25 °C in vitro, the catalytic reaction was started by the addition of substrate.

Statistical Analysis

Calculations were performed using the statistical programs 'Stat 5.3' by *Gary Perlman*, University of California, San Diego, and a non-linear regression program by *Koeppe & Hamann* (13) based on the least squares method, which was run on a Siemens MX-2 microcomputer. The concentrations required to achieve 20–80% inhibition of the enzyme activity were calculated from the fitted concentration response using an empirically modified *Hill* equation with the following set of parameters:

$$I = \frac{a}{\left(\frac{b}{c}\right)^c + 1} + d \cdot \log c + e$$

Results

Butyrylcholinesterase activity was selectively inhibited by 0.1 mmol/l tetraisopropylpyrophosphoramide in some samples of postmortem human brain cortex and hippocampus; this did not inhibit acetylcholinesterase in the assay used, as demonstrated in earlier investigations (11), or change the concentration response of total cholinesterase activity to galanthamine, physostigmine and tacrine, indicating that the catalytic activity of cholinesterase in the human brain samples was almost completely related to acetylcholinesterase activity. To avoid interactions between tetraisopropylpyrophosphoramide and a second inhibitor, the subsequent concentration-response trials were performed without specific inhibition of butyrylcholinesterase activity.

Assays of frontal cortex and hippocampal samples revealed 2- to 3-fold higher acetylcholinesterase activities in the latter, as reported by other authors (8, 14). No significant differences, however, were seen between the two regions with respect to the inhibition of acetylcholinesterase activity by galanthamine, physostigmine or tacrine (fig. 1; tab. 1). Acetylcholinesterase activity in the human brain frontal cortex tissue obtained at autopsy was inhibited over a range of 20–80% most effectively by physostigmine (3–74 nmol/l), followed by tacrine at between 210 nmol/l and 4.0 µmol/l and galanthamine at between 650 nmol/l and 17.8 µmol/l (fig. 1; tab. 1). Human brain temporal cortex tissue and the frontal cortex homogenate showed identical inhibition by physostigmine (fig. 1; tab. 2). Comparison of fresh brain cortex biopsies with postmortem cortex tissue revealed the same inhibitory response to galanthamine. Red-cell cholinesterase activity in blood samples from the neu-

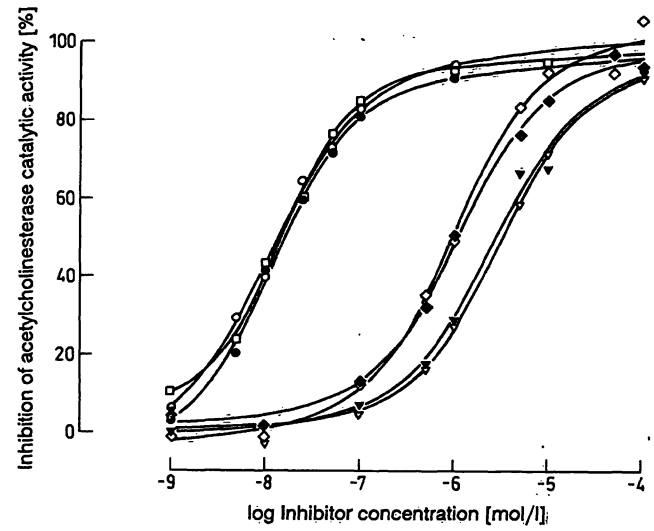

Fig. 1. Concentrations of between 1 nmol/l and 0.1 mmol/l of galanthamine (▽, ▼), physostigmine (○, ●, □), and tacrine (◇, ◆) were incubated for 1 h at 25 °C in vitro with samples of human brain cortex of the frontal (○, ◇, ▽) or temporal (●) region and human brain hippocampus (□, ◆, ▼) before the reaction was started by adding substrate. Values represent the mean inhibition in postmortem homogenate samples from 6–11 different patients at autopsy with less than 28 h delay. Mean values were derived from the averages of duplicate assays.

Tab. 1. Inhibition of acetylcholinesterase activity in human brain tissue

Tissue	n	Inhibitor	Inhibitor concentration [nmol/l]											
			1	5	10	25	50	100	500	1000	5000	10000	50000	100000
Frontal Cortex	9	Physostigmine	6.2 (2.4)	29.8 (1.6)	40.3 (2.7)	65.2 (1.2)	73.7 (2.4)	83.6 (2.2)		94.8 (2.4)				
Hippo-campus	10	Physostigmine	10.6 (2.0)	24.2 (2.7)	43.6 (2.3)	60.5 (2.8)	76.9 (1.9)	86.0 (2.5)		93.9 (1.4)		95.8 (2.0)		
Frontal Cortex	9	Tacrine	−0.7 (0.7)		−1.1 (5.1)			12.9 (2.0)	35.7 (1.9)	49.9 (2.8)	84.2 (2.9)	93.1 (3.9)	92.5 (2.3)	106 (7.6)
Hippo-campus	10	Tacrine	4.8 (3.5)		2.4 (2.9)			13.4 (1.5)	32.1 (2.6)	51.2 (2.5)	76.8 (3.1)	85.7 (1.8)	97.2 (2.5)	94.3 (1.6)
Frontal Cortex	11	Galanthamine	5.6 (4.9)		−2.3 (3.8)			4.9 (2.1)	16.7 (2.7)	27.7 (2.1)	59.0 (2.8)	71.9 (1.5)		91.5 (1.1)
Hippo-campus	10	Galanthamine	0.0 (2.5)		2.2 (2.5)			7.3 (2.8)	18.1 (2.2)	29.9 (1.4)	66.8 (2.0)	68.3 (4.0)		93.1 (1.1)

Concentration response for inhibition by 1 nmol/l to 0.1 mmol/l of physostigmine, tacrine and galanthamine, which were incubated for 1 h at 25 °C in vitro with n different samples of postmortem human brain tissue. Mean values of enzyme inhibition (% of uninhibited control) and the standard error of the mean (in brackets) are given.

rosurgery patients, however, was 10 times more strongly inhibited by galanthamine than the respective enzyme activity in brain tissue samples. This intraindividual comparison reflected the same differences as those between human postmortem brain tissue and acetylcholinesterase inhibition in red cells of 8 healthy male volunteers of an earlier trial ((12); fig. 2). In contrast to galanthamine, each of the cholinesterase inhibitors, physostigmine and tacrine, diminished enzyme activities in human brain tissue and human erythrocytes to the same degree. Concentrations of the inhibitors exerting a half-maximal effect (IC_{50} values) on acetylcholinesterase of various sources are summarized in table 2. Evaluation of acetylcholinesterase activity in the brain cortex homogenate and erythrocytes of NMRI-mice resulted in a slope of the concentration response curve, which was flatter than that in human tissues, as described above. However, the red cells and brain of NMRI-mice showed no relevant differences in acetylcholinesterase inhibition (fig. 3; tab. 2).

Discussion

In this study, physostigmine was found to be more potent than tacrine and galanthamine in suppressing cholinesterase activity in the human brain. The data correspond very well to those of a similar trial (5), in

Tab. 2. IC_{50} of various acetylcholinesterase inhibitors

Species	Group	Tissue	Region	(n)	Physo-stigmine	(n)	Tacrine	(n)	Galan-thamine
Human	Postmortem	Brain	Hippo-campus	(10)	15	(10)	1070	(10)	2750
Human	Postmortem	Brain	Frontal Cortex	(9)	14	(9)	950	(11)	3240
Human	Postmortem	Brain	Temporal Cortex	(6)	16				
Human	Neurosurgery	Brain	Cortex					(4)	2900
Human	Neurosurgery	Erythrocyte						(4)	370
Human	Volunteer	Erythrocyte		(7)	27	(8)	1590	(8)	360
Mouse	Decapitation	Brain	Cortex	(5)	63	(5)	1290	(5)	4400
Mouse	Decapitation	Erythrocyte		(5)	68	(5)	1230	(5)	5100

Physostigmine, tacrine and galanthamine were incubated for 1 h at 25 °C in vitro with n different samples of various tissues. The inhibitor concentrations (nmol/l) producing a half-maximal effect (IC_{50}) on acetylcholinesterase were derived from a plot of % enzyme inhibition versus log concentration of inhibitor.

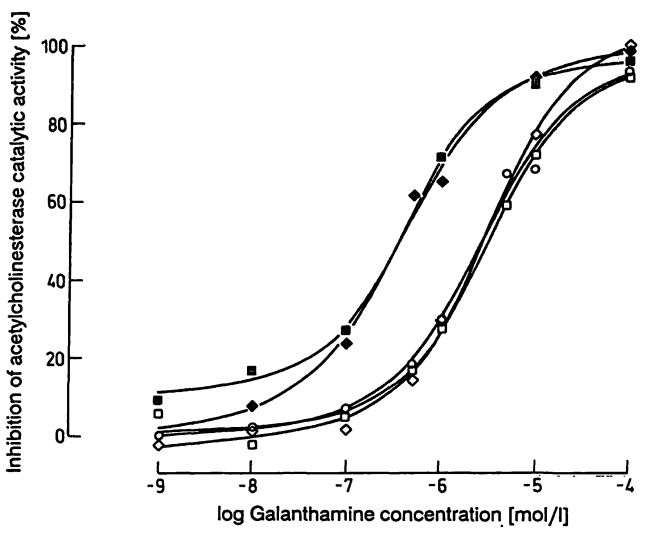

Fig. 2. Comparison of the inhibitory effects of galanthamine on acetylcholinesterase activity in human brain tissue and human erythrocytes in vitro. Human brain tissue was obtained at autopsy less than 28 h postmortem from 2 regions, frontal cortex (□) and hippocampus (○), or as fresh tissue during neurosurgical removal (◇) of a brain tumour. Human red cells were obtained from healthy volunteers (■) and the respective neurosurgical patients (◆) before the induction of anaesthesia. Assay conditions as stated in figure 1. Values represent the mean inhibition of 4 (neurosurgery group), 10–11 (postmortem group), and 7 or 8 (volunteers) samples.

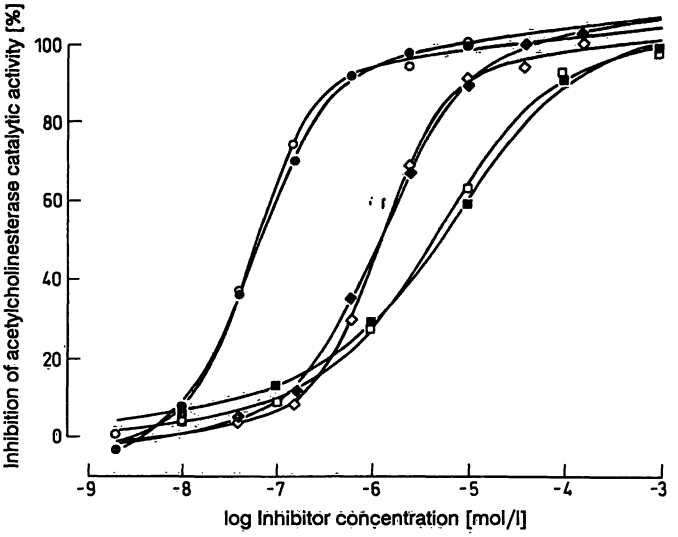

Fig. 3. Concentrations of between 0.5 nmol/l and 0.5 mmol/l of galanthamine (□, ■), physostigmine (○, ●) and tacrine (◇, ◆) were incubated for 1 h at 25 °C in vitro with samples of NMRI-mice brain cortex (open symbols) and erythrocytes (filled symbols). Values represent the mean of n = 5 different samples.

which *Perry* and colleagues used physostigmine and tacrine in samples of postmortem parietal cortex obtained after an equal autopsy delay from 3 individuals with no clinical or pathological evidence of any neurological or psychiatric disorder. There are minor differences between the two reports, i.e. they found tacrine to be 1.5 times more and physostigmine 2 times less potent than we did. Both cholinesterase inhibitors have been widely used in vivo, but relatively few studies have reported plasma/serum concentrations of physostigmine (15–17) or tacrine (18–20). The doses were increased until adverse peripheral effects in the majority of the respective patients or volunteers indicated the upper tolerance level. These concentrations were in the range of 11–25 nmol/l with physostigmine (15) and 60–220 nmol/l with tacrine (18). Peak galanthamine concentrations after intravenous application of 0.3 mg/kg body weight in anaesthetized patients were 1890 nmol/l (21), while drug concentrations in plasma 2 h after chronic oral administration of 15 mg three times daily in *Alzheimer* patients (22) were in the range of 400–550 nmol/l.

According to our data (fig. 1; tab. 1; tab. 2), these measured plasma concentrations would not result in high degrees of central cholinesterase inhibition. It has been reported, however, that galanthamine, physostigmine and tacrine accumulate in the brain tissue of small animals. If this occurs to an equal extent in humans, physostigmine might be increased 2-fold (23, 24) and tacrine 8–12-fold (25), resulting in an estimated acetylcholinesterase inhibition of 65% and 75% respectively. In the case of galanthamine (26), the accumulation factor of 2 would only lead to a 25–35% enzyme inhibition in the human brain. This finding may be explained by the fact that galanthamine, in contrast to physostigmine and tacrine, was markedly less potent in suppressing acetylcholinesterase activity in the human brain than in human red cells. One might speculate whether galanthamine separated between different molecular G-forms of acetylcholinesterase (erythrocytes dimeric; brain tetrameric), while physostigmine and tacrine did not, as was recently shown for the G_1 and G_4 form using the latter 2 compounds (27). There were no differences in enzyme inhibition by galanthamine, physostigmine and tacrine, however, between erythrocytes and brain tissue of mice.

In vitro measurement of cholinesterase inhibition in tissue homogenates may not reliably reflect true drug concentrations, true inhibition of acetylcholinesterase activity or in vivo alterations at synaptic sites in the brain. In addition, the rate of appearance and the effects of various metabolites of the cholinesterase inhibitors are still unclear. Moreover, it has been reported that, in *Alzheimer* patients, the location of cholinesterases has largely shifted to the neuritic plaques and tangles (28), giving rise to the speculation that cholinesterase-inhibiting drugs may act differently in an *Alzheimer* brain than in a non-demented one. And finally, although there is some evidence on the relationship between enzyme inhibition and

changes in acetylcholine content or turnover (25, 29, 30), the degree of acetylcholinesterase inhibition required to elicit central effects in the human brain is still unknown.

Despite all of these serious limitations, some conclusions, however, may be drawn from the current data:

1. Inhibition of catalytic enzyme activity in erythrocytes, as measured ex vivo in the patient, is a fairly close predictor of cholinesterase inhibition in the brain if physostigmine or tacrine have been given. The measurement of peripheral acetylcholinesterase activity after galanthamine application may be used as a safety parameter and estimate of central inhibition.

2. Clinical studies on the efficacy of reversible cholinesterase inhibitors should employ an individual dose finding phase according to the report of a consensus conference on the methodology of clinical trials of "nootropics" (31) and, in addition, include the concomitant degree of ex vivo enzyme inhibition. The lower doses of galanthamine, physostigmine and tacrine used so far in many clinical studies possibly did not cause the anticipated degrees of inhibition of brain acetylcholinesterase activity, and this would explain in part the limited effect.

3. Ex vivo measurement of enzyme inhibition should be performed as soon as possible during clinical investigation to verify the in vitro findings.

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